The Effect of Age and Sex on the Number and Osteogenic Differentiation Potential of Adipose-Derived Mesenchymal Stem Cells

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The Effect of Age and Sex on the Number and Osteogenic Differentiation Potential of Adipose-Derived Mesenchymal Stem Cells

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SUMMARY

It has been shown that stem cells exist within adult adipose tissue. These stem cells are named adipose-derived mesenchymal stem cells (ASCs), are derived from the mesoderm, and can differentiate into a number of cells including osteoblasts, chondrocytes, and adipocytes. However, before these cells can be used clinically it is important that we understand how factors like age, sex, and ethnicity affect ASC number and potential. Additionally, since men and women vary in their distribution of adipose tissue, it will be important to see if the ideal source of ASCs is different for each sex. The goal of this study was to assess how age and sex affects ASCs. We used flow cytometry to investigate how age and sex affected the number of ASCs in adipose tissue. Additionally, we plated these cells in culture and treated them with an osteogenic media (OM) with the intention of pushing them towards osteoblast differentiation. The purpose of this was to see if age or sex affected the potential of the ASCs to undergo osteogenesis in culture. For this study we used real-time PCR and biochemical assays to look at markers of early and late osteogenic differentiation. Finally, we used immunohistochemistry to demonstrate where in adipose tissue the CD73 and CD271 positive cell population exists. It is our hope that this work will shed light on how age and sex affect ASCs so that clinicians can optimize their ASC harvest depending on the patient's physiology.

Methods: Adipose tissue was harvested from the inguinal fat pad of 1M, 3M, and 9M male rats, and 3M and 9M female rats. The tissue was washed several times and then digested with trypsin, collagenase, and dispase. After the isolation procedure we were left with the stromal vascular fraction containing ASCs as well as other cell sources like fibroblasts, red blood cells, and endothelial cells. Using flow cytometry we assessed the primary cells for the number and percentage of ASCs and osteoprogenitor cells (OPCs). We used a combination of antibodies

to denote a cell as either an ASC or OPC. ASCs were CD73 and CD271 positive, and CD45 negative. OPCs were osteocalcin (OCN) and E11 positive. We also paraffin embedded adipose tissue and stained 5um slices with CD73/CD271, von Willebrand Factor, hematoxylin and eosin, or a control antibody to see where our ASC population exists. Additionally, we plated ASCs on tissue-cultured polystyrene and grew them in a mesenchymal growth media (GM) or OM for 7 and 14 days. At these time points we harvested the cells and looked at markers of osteogenesis. We ran biochemical assays looking for the activity and levels of alkaline phosphatase (ALPase), OCN, and osteoprotegerin (OPG). We also ran real-time PCR to look for the expression of RUNX2, ALPase, OCN, and Bone Sialoprotein (BSP).

Results: From the initial flow cytometry we discovered that the number and percentage of ASCs decreases as the animal gets older. On the other hand, sex did not have an effect on the number of ASCs. We also discovered that the percentage of OPCs increases as the animal gets older. When we compared the sexes, there was no difference at 3M, but at 9M the females had more OPCs. We also noticed that the young and old animals had different tissue morphologies. The younger animals had white adipose tissue while the older animals had brown adipose tissue. The histology showed that the CD73+/CD271+ cells are localized to the vasculature and we saw co-localization of the antibodies in the capillaries and veins. When the cells were cultured in OM they all expressed markers of osteogenesis indicating that they had a similar potential for osteogenesis. However, differences were seen between the ages and sexes. The younger cells proliferated at a greater rate than the older cells in the GM. Additionally, the older cells secreted and expressed more OCN. The major difference between the sexes is that the males decreased their secretion of OPG in the OM while the female cells maintained similar levels in both medias.

Discussion: The results indicate that in older age the adipose-derived number of ASCs decreases and the number of OPCs increases. Although our technique to examine ASC and OPC number is unique, the results agree with other studies showing that the number of adult stem cells decreases with age. This is important because it indicates that retrieving ASCs from older patients may require more tissue and/or pretreatment to increase the number of stem cells. Furthermore, the results indicate that the osteogenic potential of ASCs does not decrease with age. This is interesting because the ability to regenerate damaged tissue does decrease with age. This would imply that the decrease in tissue regeneration could be attributed to a lowered stem cell number, or as other researchers have discovered, the cues required to trigger stem cell migration are altered with age.

The results of the sex study indicate the neither ASC number nor osteogenic potential changes between the sexes. These results disagree with other studies showing that male ASCs have a higher osteogenic potential than female ASCs. The difference between our studies is the animal model and tissue source. This is important because the ideal site to harvest ASCs may be different between men and women, and so it will be important for future investigators to examine that possibility.

CHAPTER 1

INTRODUCTION

Adipose Tissue as a Source of Mesenchymal Stem Cells

It was demonstrated in 2001 that multipotent stem cells exist within adipose tissue (Zuk et al., 2001). Since then research has shown that these stem cells are similar in the immunophenotype and differentiation potential as the mesenchymal stem cells found within the bone marrow (Kern *et al.*, 2006; Liu *et al.,* 2009). Therefore these adipose-derived mesenchymal stem cells (ASC) are an attractive source for regenerative medicine because they can differentiate into many tissue lineages including osteoblasts, chondrocytes, and adipocytes. Using adipose tissue as a source of stem cells is advantageous over other sources for several reasons. As a source of adult stem cells, working with adipose tissue avoids the ethical dilemma associated with embryonic stem cells. Additionally, adipose tissue is easier to isolate than most stem cell sources (especially compared with bone marrow), it is abundant in humans, and the stem cells proliferate rapidly in culture. Furthermore, several studies have shown that ASCs do not express the major immunologic surface antigens and therefore do not elicit an allogenic immune response (Niemeyer et al., 2007). Due to these findings this young field of ASCs has blossomed over the past decade as researchers continue to study the differentiation potential and regenerative capacity of these cells.

While much attention has been given to ASCs over the past decade, many questions need to be addressed before these cells can be used clinically. Some of the main questions deal with how age, sex, ethnicity, and anatomical source affect the number and differentiation potential of ASCs. It has already been shown that changes in some of these variables will affect the potential

of ASCs to differentiate. One study compared visceral and subcutaneous ASCs from rabbits and discovered that while subcutaneous ASCs proliferated more, ASCs from visceral adipose tissue had a higher osteogenic potential (Peptan et al., 2006). This was attributed to the cellular makeup and vasculature of the adipose tissue. Since the cells isolated from adipose tissue have a heterogeneous phenotype, the cells from one adipose depot may differ from the cells in another depot. The two variables that this paper focuses on are how the age and sex of the donor affects the number of adipose-derived mesenchymal stem cells and their osteogenic differentiation potential. These questions are important to answer because the results will enable researchers to optimize their patient's ASC treatment depending on the donor's source of adipose tissue, age, sex, etc. It is possible that each patient may require a treatment that is tailored to his/her physiology and needs.

Role of Aging in Stem Cells

Our regenerative capacity has been shown to decrease as we age, especially our ability to repair injuries to our musculoskeletal system (D'lppolito et al., 1999; Fisher et al., 2009). A few possible explanations are that as we age we lose the number of stem cells in our reservoir, the ability of these to cells to undergo differentiation decreases with age, and that the extrinsic factors required to stimulate stem cell migration and differentiation are altered with age. Some studies have shown a decrease in gene expression with age, leading to a decline in stem cell migration, adhesion, and protection from DNA damage (Wagner *et al.,* 2009; Kasper *et al.*, 2009). Several studies have investigated the effect of aging on ASCs osteogenesis, but the results have been conflicting. This is partially due to the lack of uniformity in ASC studies. While human adipose tissue is the ideal source for studying ASCs, it is difficult to acquire this tissue from multiple patients with a similar age, gender, and anatomical source of extracted

adipose tissue. Furthermore, animal studies differ in their isolation method, culture medium, and biochemical assays used to quantify number of stem cells or level of differentiation. A study by Shi *et al.* (2004) observed that osteogenic potential was maintained with aging, while another study by Kasper *et al.* (2009) reports that the osteogenic potential of ASCs can decrease with age. Whatever the differences may be attributed to, it is likely that a difference in aged ASCs, whether intrinsic or extrinsic, does exist, and it will be important to elucidate the age related changes so that we can optimize future stem cell therapies. The advantage of the study being presented here is that the researcher has investigated both stem cell number and osteogenic potential. By addressing both the number of ASCs and osteoprogenitor cells (OPCs) present in primary tissue, as well as the osteogenic potential in culture, a more complete picture can be created on the potential of stem cells from adipose tissue. Additionally, while most studies have focused on histological assays for osteogenesis, this study includes gene studies and biochemical assays. The advantage of this method is that it will not only address differences in osteogenic potential between ASC sources, but also help researchers understand the causes of these differences.

Role of Sex in Stem Cells

Another factor that this paper investigates is how the sex of the individual affects their ASC potential. It is important to investigate the role of sex on ASCs because the amount and distribution of adipose tissue between men and women is not the same (Freedman et al, 1990). This is due to a hormonal difference between men and women that develops as they reach puberty leading to contrasting levels of testosterone and estrogen (Guyton and Hall, 2000). For this reason, it may turn out that the optimal site to harvest ASCs is different between men and women. Additionally, the hormonal balance between men and women changes throughout their

lives, and therefore the microenvironment of the ASC will alter as the individual ages. For example, women experience a drop in estrogen levels as they reach menopause (Luftkin et al, 1992). This decline in estrogen leads to a reduction in osteoblastic activity and an increased susceptibility to bone disorders like osteoporosis (Riggs et al., 1998). Since estrogen can be metabolized in adipose tissue by conversion of androgen via aromatase, it may turn out that a difference in the level of adipose-derived estrogen, as well as the response to this steroid, can affect the ability of ASCs to differentiate into osteoblasts (Simpson et al., 1981). Therefore, the difference in hormone levels between men and women may affect their ASC differentiation potential, and this potential may continue to change as men and women age and their hormonal balance alters. Additionally, any differences in the ASC phenotype between men and women may cause the cells to react differently *in vitro*. By addressing both the initial ASC and OPC populations, as well as the osteogenic potential of ASCs in culture, it is the hope of the researcher to shed some light on the differences, if any, between the two sexes.

The Use of Flow Cytometry in Addressing the Primary ASC and OPC Populations

The initial goal of the researcher was to quantify the number of ASCs and OPCs isolated from the stromal vascular fraction of adipose tissue per gram of fat. To achieve this, the researcher used flow cytometry and denoted a positive population based on the cell's immunophenotype. Unlike embryonic stem cells, no specific markers for ASCs exists, so therefore a combination of markers were used to pinpoint a specific cell type. The ASCs were declared as being CD271 positive, CD73 positive, and CD45 negative. CD45, the common leukocyte antigen, was used to eliminate the population of cells from the hematopoietic lineage, which in some cases included up to 20% of the isolated cells. CD45 has been found on the surface of cells from the haematopoietic lineage, but not on the surface of ASCs (Schaffler et al.,

2007). We used CD271 and CD73 as positive markers of ASCs because previous research has shown that adipose-derived cells positive for these markers can be pushed towards osteogenesis when given the right media (Mitchell et al., 2006; Astori et al., 2007; Quirici et al., 2002; Barry et al., 2001). CD271, low-affinity nerve growth factor receptor, was originally identified in a population of bone marrow cells that stained positive for alizarin red *in vitro* (Quirici *et. al,* 2002). Since then this protein has been found on the surface of adipose-derived cells that undergo osteogenesis in culture (Quirici *et. al,* 2009). Although the exact role of this marker in ASCs is not known, it is thought to present growth factors involved in morphogenesis. CD73, ecto-5' nucleotidase, was also originally described as being present on the surface of bone marrow cells (Barry *et. al,* 2001). This phosphatidylinositol-anchored protein is thought to be involved in signal transduction and dephosphorylation of purines and pyrimidines into nucleosides. Current research has shown that adipose-derived cells positive for this marker demonstrate an osteoblast phenotype when treated with induction media in culture. (Leong *et al.,* 2006). Based upon these studies and others, we believed that using our three markers in conjunction with one another allowed our group to compare the number and percent of ASCs across different ages and sexes.

The other cell type that was investigated in primary tissue and in culture was an OPC. The OPC is a MSC that has started to differentiate towards the osteoblast lineage, usually seen by an increase in RUNX2 and alkaline phosphatase protein levels. The two surface markers used to identify this cell type were E11 and osteocalcin (OCN). E11, podoplanin, is a hydrophobic glycoprotein that is necessary for the elongation of the dendritic processes in osteocytes (Zhang *et al.,* 2006). It has been noted that ASCs will increase their dendritic processes needed for cellular communication towards the end of osteogenic differentiation, and this increase has been

correlated with an increase in E11 expression (Elabd *et al.,* 2007). We used E11 as a marker to identify the cells in primary tissue that have started to differentiate into an osteocytes, and the cells in culture that have formed the phenotype associated with osteocytes. The other marker we used, OCN, is a well-known marker associated with end stage osteogenesis. This small protein is secreted by osteoblasts and plays a role in mineralization and calcium-ion homeostasis by binding calcium in the extracellular matrix. Even though cells secrete OCN, it was shown that this protein could be identified on the cell's surface using flow cytometry (Eghbali-Fatourechi et al., 2007). The reason is that either OCN uses its Gla residues to temporarily bind the cell surface as it's secreted, or that OCN binds to an OCN receptor. This evidence has indicated that OCN is another marker that can be used in extracellular flow cytometry to define a population of cells displaying an osteoblast phenotype and having undergone later differentiation. It is believed that a cell positive for E11 and OCN has left the multipotent stem cell state and has started to undergo osteogenesis, or is already a mature bone cell.

The researcher used flow cytometry to determine both the percentage of cells positive for the markers listed above and also to figure out the number of cells per gram of adipose tissue positive for these markers. The purpose of this second goal was to gather a more clinically relevant number. For example, even if the percentage of ASCs is higher in one population, if the total tissue or cellular yield is lower, then the total number of ASCs might also be lower. Given this information, a clinician can more easily determine the amount of adipose tissue necessary in order to achieve a desired number of ASCs. Therefore, the researcher correlated the percent of ASC and OPC positive cells, with the number of cells per gram of adipose tissue, to extract the number of ASCs and OPCs per gram of fat. The equation is given below:

$$\frac{\text{Total Number of Cells}}{\text{Grams of Adipose Tissue}} \times \frac{\text{\% of Positive Cells}}{100}$$

Correlating Rat and Human Ages: Rationale Behind the Ages We Chose

To study age we used only male rats that were 4 weeks, 12 weeks, and 36 weeks old upon arrival. We focused on these ages because they correlate with humans that are in different stages of maturation (Fisher et al., 2009). At 4-weeks the rats have not yet reached sexual maturity and so they can be compared to a human who is somewhere between childhood and early adolescence (Quinn et al., 2005). At 12-weeks the rats have reached sexual maturity and they are also continuing to grow, so these animals can be considered adults. Finally, at 36-weeks the rats have stopped growing but unlike humans there is no epiphyseal closure in the long bones (Harlan Growth Chart[1]). Due to these circumstances our group considers the 36-week rats as an aged population.

In addition to looking at the effect of age on ASCs, we also investigated the role sex plays. We chose to focus our study on 3M and 9M male and female rats. We chose these ages because at 3M the rats are just starting their sexual maturity, and at 9M they have stopped their skeletal development. Due to these circumstances, we believe that the adipose microenvironment and hormonal balance would be most different at these ages.

1 http://www.harlan.com/research_models_and_services/research_models_by_product_type/outbred_rats/sprague_dawley_sd

SPECIFIC AIMS AND EXPERIMENTAL DESIGN

The **overall goals** of this thesis are to investigate the effects age and sex have on the number of adipose-derived stem cells in the inguinal fat pad, and to study the effects age and sex have on the osteogenic differentiation potential of these ASCs. The **general hypothesis** is that age, but not sex, will affect the number of ASCs in the inguinal fat pad. Furthermore, we believe that both age and sex will affect how these cells respond to our osteogenic media, and therefore the osteogenic differentiation potential of these cells.

Aim 1: To study the effect age has on ASCs; this was divided into three sub-aims.

Aim 1.1: To investigate how age affects the number of ASCs in the inguinal fat pad. The **objective** of this aim was to study the number of ASCs and OPCs in the inguinal fat pad of 1M, 3M, and 9M male Sprague-Dawley rats. We achieved this by staining our cells with antibodies of extracellular markers of ASCs and OPCs. By using a combination of antibodies we were able to extract the ASC and OPC percent and number using flow cytometry. Our **hypothesis** was that the older animals would have a lower percent and number of ASCs. We believed this to be the case since older animals have a decreased ability to regenerate their tissue, and a reduction in the number of stem cells could be a reason why.

Aim 1.2: To discover the location of CD73 and CD271 positive ASCs. The **objective** of this aim was to use histological methods on adipose tissue to find cells positive for both of our ASC markers. We paraffin embedded adipose tissue from 6-week males and stained the tissue slices with antibodies for CD73/CD271 or vWF. We then stained them with a secondary fluorescent antibody and visualized the histology on a light microscope with a camera attached. Our **hypothesis** is that the ASCs would be found in or around the vasculature. We believed this to be

the case because the vasculature is a large component of the stromal vasculature fraction that we isolate from the adipose tissue.

Aim 1.3: To investigate how age affects the osteogenic differentiation potential of ASCs from the inguinal fat pad. The **objective** of this aim was to study the osteogenic differentiation potential of ASCs in culture on TCPS. We plated 1M, 3M, and 9M male Sprague-Dawley rat ASCs on TCPS and fed them with GM and OM for 7 and 14 days. At these time points we used flow cytometry, Real-Time PCR, and biochemical assays to study their expression and level of some common markers of osteogenesis. Our **hypothesis** was that the older animals would have a decreased potential for osteogenesis. This was our rationale because older animals have a decreased ability to repair damaged tissue, and also some studies have shown that MSCs from older animals have a decreased ability to undergo osteogenesis.

Aim 2: To study the effect sex has on ASCs; this was divided into two sub-aims.

Aim 2.1: To study the role sex has on the number of ASCs in the inguinal fat pad. The **objective** of this aim was to study the number of ASCs and OPCs in the inguinal fat pad of 3M and 9M male and female Sprague-Dawley rats. We achieved this by staining our cells for markers of ASCs and OPCs and then using flow cytometry to figure out the number and percent of these cells. Our **hypothesis** was that sex would not affect the number of ASC and OPCs. We believed this to be the case since the animals are of a similar age and should have equal abilities to regenerate tissue. Therefore, sex should not be a factor in this process.

Aim 2.2: To investigate how sex affects the osteogenic differentiation potential of ASCs from the inguinal fat pad. The **objective** of this aim was to study the osteogenic differentiation potential of 3M and 9M male and female rat ASCs in culture on TCPS. We plated these cells on

TCPS and fed them with either a GM or an OM for 7 and 14 days. At these time points we used flow cytometry, Real-Time PCR, and biochemical assays to study their ability to undergo osteogenesis. Our **hypothesis** was that the female ASCs would have a decreased ability to differentiate. Our rationale behind this is that older females experience more bone turnover, an increase in osteoclastogenesis, and suffer more from conditions like osteoporosis. We believe that differences in the female ASC caused by exposure to their unique environmental niche may cause a reduction in the potential of their ASCs to undergo osteogenesis.

METHODS

Tissue Dissection

Sprague Dawley rats were purchased from Harlan Laboratories for this study. We examined male rats that were 4 weeks, 12 weeks, and 36 weeks old upon arrival for the first aim. For the second aim we used male and female rats that were 12 weeks and 36 weeks old upon arrival. For each study the animals were dissected and the cells harvested concurrently to maximize the number of comparisons possible. The Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology approved the study. The rats were euthanized with CO_2 and then placed on an operating board. The legs were washed with ethanol and betadine two times, and sterile surgical gauze was used to remove any remaining solution. Using autoclaved scissors the initial incision was made from the ankle to the torso, exposing the inguinal fat pad of both legs. After the incision adipose tissue was removed and then immediately placed in a 50mL conical tubes with 10mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 3% sterile-filtered L-Glutamine-penicillin-streptomycin (P/S). The 50mL conical tubes were weighed before and after the addition of adipose tissue.

Cell Isolation

After the dissection, the adipose tissue was then brought under a sterile laminar flow hood and washed three times with 10mL of DMEM + 3% P/S and two times with 15mL of phosphate buffered saline (PBS) with 1% P/S. After the second wash the PBS was removed, and the tissue was incubated on a rocking platform shaker at 37^{o}C in 5mL of trypsin for 30 minutes. After the incubation the trypsin was quenched with 10mL of DMEM with 10% fetal bovine serum (FBS) and 1%P/S. The tissue was then secured with a hemostat and cut directly into a new 50mL conical tube with scissors. The adipose tissue was then treated with 15mL of our digestion cocktail which included sterile filtered DMEM + 1% P/S supplemented with collagenase type I (365units/mL) and dispase (3units/mL). The conical tube was then incubated on a rocking platform shaker at 37^{o}C for three hours. After the digestion the cell suspension was transferred through a 40μm cell strainer into a new conical tube, and the reaction was quenched with 15mL DMEM + 10% FBS + 1% P/S (Full Media). The cells were then spun down three times in a centrifuge at 2000 RPM for 10 minutes. Following each spin, the supernatant was aspirated and the cells were washed with PBS + 1% P/S. After the last wash the number of cells was counted on a Beckman Coulter Z-1 particle counter. Two hundred thousand cells from each group were plated in a V-bottom 96-well plate for flow cytometry. The remaining cells were grouped together by age, spun down at 2000 RPM for 5 minutes, and then recounted. The cells were plated on a tissue-cultured polystyrene (TCPS) T-75 at 5,000 cells/cm^2 and fed with 10mL of Mesenchymal Stem Cell Growth Media (Lonza, GM, PT-3001). Each group had 5 T-75s with 375,000 cells/flask.

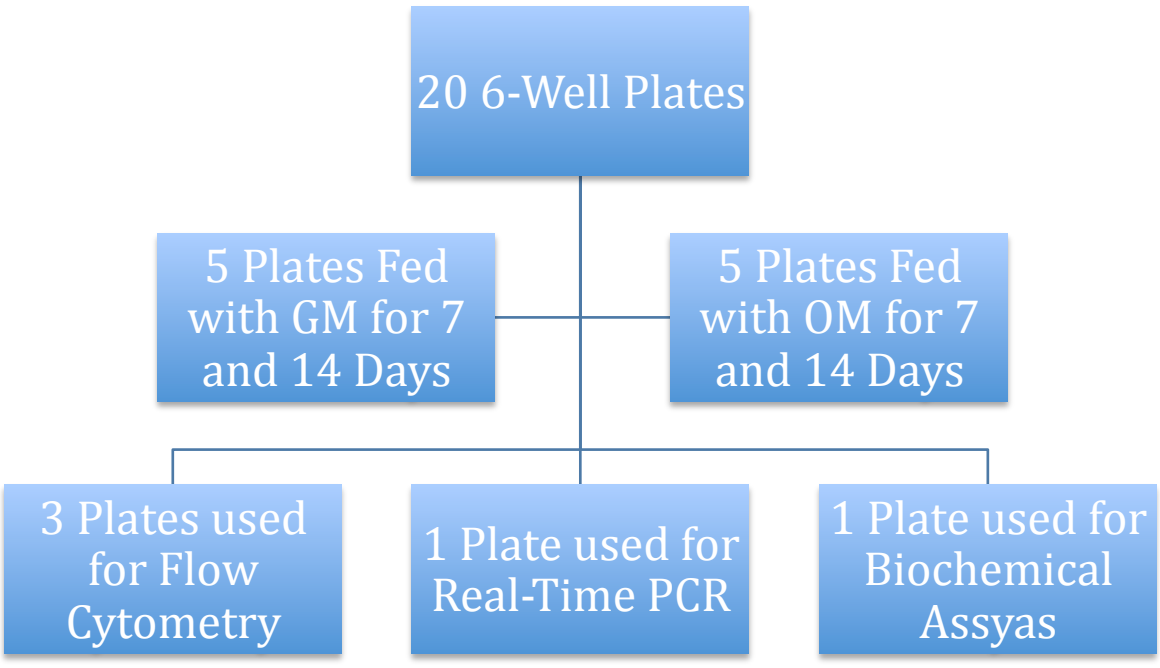
Histology

Adipose tissue from the inguinal fat pad of 6-week old male Sprague-Dawley rats was fixed with 10% neutral buffered formalin and then paraffin embedded. 5µm tissue slices were prepared. The tissue was deparaffinized and select samples were processed for Hematoxylin and Eosin staining. Antigen retrieval was performed on the remaining sections using a pressure cooker with the samples incubated in sodium citrate buffer pH 4.2. The samples were blocked with 10% normal goat serum in PBS for 60 minutes. Immunostaining was performed using a monoclonal CD271 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, SC-71691) conjugated to Alexa Fluor 488 (Invitrogen, A11001), a polyclonal CD73 antibody (Santa Cruz Biotechnology, SC-25603) conjugated to Alexa Fluor 594 (Invitrogen, A11012), and a polyclonal Von Willebrand Antibody (vWF, Abcam, Cambridge, MA, AB6994) conjugated to Alexa Fluor 594. The primary antibodies were incubated on the samples overnight at 4^{o}C while the secondary antibodies were on the samples for 60 minutes. vWF was used as a positive marker for the vasculature because it is expressed in endothelial cells [47]. One section was stained for both CD271 and CD73 and another section was stained for vWF. The negative controls used were a rabbit IgG (Thermo Fisher Scientific, NC-100-P) conjugated to Alexa Fluor 594 and a mouse IgG (Santa Cruz Biotechnology, SC-2025) conjugated to Alexa Fluor 488. Images were taken on an ORCA-ER digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan, C4742-80-12AG) attached to a Leica DMLB microscope (Leica, Wetzlar, Germany, DFC420), and the images were processed using SimplePCI 6 software (Compix Inc. Imaging Systems, Sewickley, PA).

Cell Culture

The cells were kept in a T-75 until 90% confluence, fed with GM every two days, and then passaged into 6-well plates. Each group of 5 T-75s was divided among 20 plates at a seeding dentistry of 5,000 cells/cm^2. A day after passage the wells were washed with PBS to remove the non-adherent cells, and the remaining cells were fed with either GM or a Mesenchymal Stem Cell Osteogenic Differentiation Medium (Lonza, OM, PT-3002). This was counted as day 1 of cell induction. The 20 6-well plates were broken into four groups of 5 and treated with either 2mL of GM or OM every two days and harvested at 7 or 14 days. 24 hours before harvest the media was changed, and 12 hours after that the RNA was collected as described below. At each harvest time point the remaining cells were used as described below for the biochemical assays.

Table 1 – Outline of Cell Culture Methods



Flow Cytometry

Flow cytometry was run on the primary cells after isolation, and on the cells immediately following the 7 day and 14 day harvests. After the cell number was counted, 200,000 cells were aliquot to a V-bottom 96-well plate (BD Bioscience 353263). The cells were stained for cell surface markers of mesenchymal stem cells, osteoprogenitor cells, or as a negative control. The mesenchymal stem cells were denoted as CD73+, CD271+, and CD45-. The osteoprogenitor cells were determined to be E11+ and OCN+. The negative controls were either secondary antibodies alone, or control isotype antibodies. A comprehensive list of the antibodies and staining steps is listed below. For the primary cells, the six rat cells were kept isolated throughout the tissue digestion. After the cells were counted, the 200,000 were added to two groups, mesenchymal stem cells and osteoprogenitor cells, for an n of 6. The remaining cells were combined and then 200,000 cells were added to another 6 wells as the controls. For the cells in culture, 3 plates were cultured for each group, one for MSCs, one for OPCs, and one for the controls. The cells were washed 3 times in 200μL of 1% PBS with BSA and stained for 30 minutes at room temperature on a VWR rocking platform. Between washings the cells were spun down at 2000 RPM for 5 minutes, and the supernatant was aspirated. After the last stain the cells were washed three times with 200μL of 1X PBS and added through a 40μm cell strainer to a TCPS Round Bottom Test Tube (VWR 60819-310). The samples were run through a BD LSR Flow Cytometer, and the data was recorded with the BD Bioscience FACSCalibur software. The cells were recorded as positive if they exceeded the negative control 5% threshold. For the primary cells the percent positive was normalized to cell number and fat weight to get the total number of positive cells per gram of adipose tissue.

Table 2 – Flow Cytometry Antibodies

Flow Cytometry Antibodies and Controls

Mesenchymal Stem Cell Markers

Antibody	Company	Moniker	Addition Step
Mouse Anti-Rat Ecto-5'-Nucleotidase	BD Pharmigen 551123	CD73	1
FITC Mouse Anti-Rat Leukocyte Common Antigen	BD Pharmigen 554877	CD45	3
PE Goat Anti-Rat NGFR P75 (C-20)	Santa Cruz SC-6188PE	CD271	3

Osteoprotegitor Cell Marker

Antibody	Company	Moniker	Addition Step
Rabbit Anti-Rat Podoplanin (KS-17)	Sigma P2120	E11	1
Goat Anti-Rat Osteocalcin (V-19)	Santa Cruz sc-18319	OCN	1

Secondary and Negative Control

Antibody	Company	Type	Addition Step
APC Goat $F(ab')_2$ Anti-Mouse IgG	R&D F0101B	CD73 Secondary/Control	2
Mouse IgG1-FITC Isotype Control	BD Bioscience 550616	CD45 Isotype Control	3
Goat IgG1-PE Isotype Control	Santa Cruz SC-3993	CD271 Isotype Control	3
FITC Chicken Polyclonal to Rabbit IgG	Abcam ab6825	E11 Secondary/Control	2
APC Donkey Anti-Goat IgG	R&D F0108	OCN Secondary/Control	2
1% PBS with BSA	Sigma	Blank	3

RNA Extraction and Real-Time PCR

Twelve hours after the last media change one plate from both the GM and OM groups were used for real-time PCR. After the media was aspirated the cells were extracted using Trizol Reagent (Invitrogen 15596-018) and stored in 15mL conical tubes kept in the -80^{o}C freezer. The lysed cells were kept in the -80^{o}C until the final harvest so that the cells from all 24 wells could be reverse transcribed at the same time. The RNA was extracted using the guanidinium thiocyanate-phenol-choloform extraction method, and the RNA concentration and A260/280 purity was recorded using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). A microgram of RNA was reverse transcribed into cDNA using an Omniscript RT Kit (Qiagen 205113) and Random Primers (Promega C1181). The cDNA was then analyzed for genetic expression by real-time PCR using the Bio-Rad Thermal iCycler. Ten nanograms of cDNA were mixed with the IQ SYBR Green Supermix (Bio-Rad 170-8882) and underwent 40 cycles of amplification. The transcript levels were quantified relative to a standard curve of known concentration, and the results were normalized to the transcript levels of the housekeeping gene GAPDH. The genes, primer information, and annealing temperatures are listed below. All primers except Osteocalcin were designed using the Beacon Designer 7.0 program and then created by the company Operon. Osteocalcin was ordered through the Qiagen Global Gene program and only the amplicon size and annealing temperature information was provided.

Table 3 – Real-Time PCR Primers

Gene	Type	Sequence	Accession Number	Amplicon Size	Annealing Temperature
Alkaline Phosphatase	R	CCT GCC TCC TTC CAC TAG C	NM_013059	101	50 – 60°C
	F	ACA ACC TGA CTG ACC CTT CC			
Bone Sialoprotein II	R	AAT AAT CCT GAC CCT CGT AGC	NM_012587	130	59.5°C
	F	TAC AAC ACT GCG TAT GAA ACC			
GAPDH	R	CAT ACT CAG CAC CAG CAT CAC C	NM_017008	121	54.1°C
	F	AAG TTC AAC GGC ACA GTC AAG G			
Nucleostemin	R	TGC CCA GGT GAC ATC TCC	NM_175580	155	55 – 60°C
	F	AAC AGA CAG AAG ACC AAC AGG			
Osteocalcin	R	Global Gene	NM_013414	113	53.9°C
	F	Qiagen– QT01084573			
RUNX2	R	AGA GGC AGA AGT CAG AGG	XM_346016	121	60°C
	F	TCC CCA TCC ATC CAT TCC			

Biochemical Assays

After culturing the cells for 7 and 14 days the media and cells were used for our biochemical assays to determine the level of osteogenesis. 1mL of media was placed in a 1.5mL eppendorf tube and stored in the -20^{o}C freezer. The cells were extracted with trypsin, counted in the particle counter, and stored in a 13 x 100mm borosilicate glass culture tube (VWR) suspended in 1mL of 0.05% of Triton-X. After the 14-day harvest the cells were sonicated for 10 seconds at 40mA and the alkaline phosphatase specific activity was measured through a colormetric assay. The results were normalized to total protein with the use of a Micro BCA Protein Assay Kit (Pierce 23235). The media was used for a radioimmunoassay to determine the level of osteocalcin protein (BTI BT-440) and a sandwich E.L.I.S.A. to determine the level of osteoprotegerin protein (R&D DY459) secreted in the media. The total count was normalized to the cell number that was recorded at harvest.

Statistics

Statistical analysis of the results was made using a General Linear Model (GLM). Difference between the groups was determined by a multiple-comparison Tukey *post hoc* test. All graphs show the mean +/- SEM and the level of significance was set at $P < 0.05$.

CHAPTER 2

THE NUMBER OF ASCs IN THE INGUINAL FAT PAD, BUT NOT THEIR OSTEOGENIC POTENTIAL, IS AGE DEPENDENT

INTRODUCTION

Even though stem cells hold potential as the future of regenerative medicine, a great deal of work still needs to be done before these cells can reach their full clinical potential. Extensive research has shown that the ability to repair damage to our skeletal system decreases as we get older, yet we are still trying to discover the underlying mechanisms for this phenomenon. Figuring out why we lose this capability with age is important because any future stem cell therapy will ultimately depend upon the host's system. Even if we prove that these ASCs can be used to repair damaged tissue, if the donor's stem cell number is too small, if the stem cells don't have the potential to repair tissue, or if the donor's bone environment doesn't send out the chemical signals require to initiate the healing process, then the therapy will ultimately be a failure. Therefore, it is imperative that we have a firm understanding of how age affects stem cells before we know how to implement any stem cell based therapy. Additionally, no studies have definitively shown where the ASCs residue within adipose tissue. The location of the ASCs will affect the chemical signals they are in contact with, and this can help shed light on their properties in relation to age.

The approach our group has taken with this study is to investigate how age affects ASCs from 1M, 3M, and 9M male rats. These ages correlate to a pre-adolescent, adult, and older population. By using flow cytometry we hope to assess how age affects the number of stem and progenitor cells present in the inguinal fat pad, a source for these adult mesenchymal stem cells. We used immunohistochemistry to study where in the adipose tissue our CD271+/CD73+ ASC population residues. Furthermore, our *in vitro* studies allow us to monitor and access the osteogenic potential of

these ASCs in a 2D environment. This research will allow us to understand what challenges future researchers might face when dealing with an aged population. The results will allow clinicians to take precautionary steps to ensure that every patient, no matter their age, is provided with the right number of ASCs under the appropriate condition so that their regeneration can be successful.

RESULTS

The result of this study shows that the percentage and number of ASCs and OPCs in male rat adipose tissue is age dependent, but that their osteogenic potential is not. The flow cytometry data shows that as age increases the number of ASCs decrease but the number of OPCs increases. Therefore, 1M rats had significantly more ASCs than 9M rats, whereas the 9M rats had significantly more OPCs than the 1M rats. The histology indicates that our ASCs reside in the vasculature. We found co-localization of CD73 and CD271 around both capillaries and veins within adipose tissue. Additionally, this study also shows that the osteogenic potential of these isolated cells *in vitro* is not age dependent. The ASCs in the osteogenic media produced a higher expression and level of osteogenic markers. This increase was significant over the RNA expression and protein levels of cells in the control growth media. While the increase was significant over the control media, no clear significance was seen between any of the age groups. Even though the different aged cells had the potential to undergo osteogenesis, there were differences in how the cells responded to the media. The older cells did not proliferate as greatly in the GM. Their production of OCN was increased over the younger cells in the OM.

Histology

The ASCs are localized to the vasculature of adipose tissue

When the adipose tissue was stained with CD73 and CD271 we saw co-localization of these markers on the cells near the vasculature (Fig. 1A,B). In Fig. 1A we can see the co-localization indicated by the yellow color around the capillary, and in Fig. 1B we can see co-localization around both a capillary and a vein. To demonstrate that the CD73/CD271 positive cells were around the vasculature we stained adjacent tissue slices with H&E as well as vWF, an endothelial specific marker. The tissue was mapped out so we could locate the same sites in multiple slides, therefore all the different images in Fig. 1A and 1B show the same vasculature but at a different depth in the adipose tissue. The H&E in Fig. 1A shows a capillary filled with red blood cells, and the H&E in Fig. 1B indicates a capillary below a vein. In both Fig. 1A and 1B the tissue is positive for vWF indicating that our images are of endothelial cells. Our controls are shown in Fig. 1C/D. Fig. 1C is a rabbit IgG control antibody for CD73 which was also raised in rabbits. Fig. 1D is a CD73 antibody that is specific for humans and was raised in a mouse. This is our control for CD271. Overall, the histology indicates that the ASCs are localized to the vasculature niche of adipose tissue.

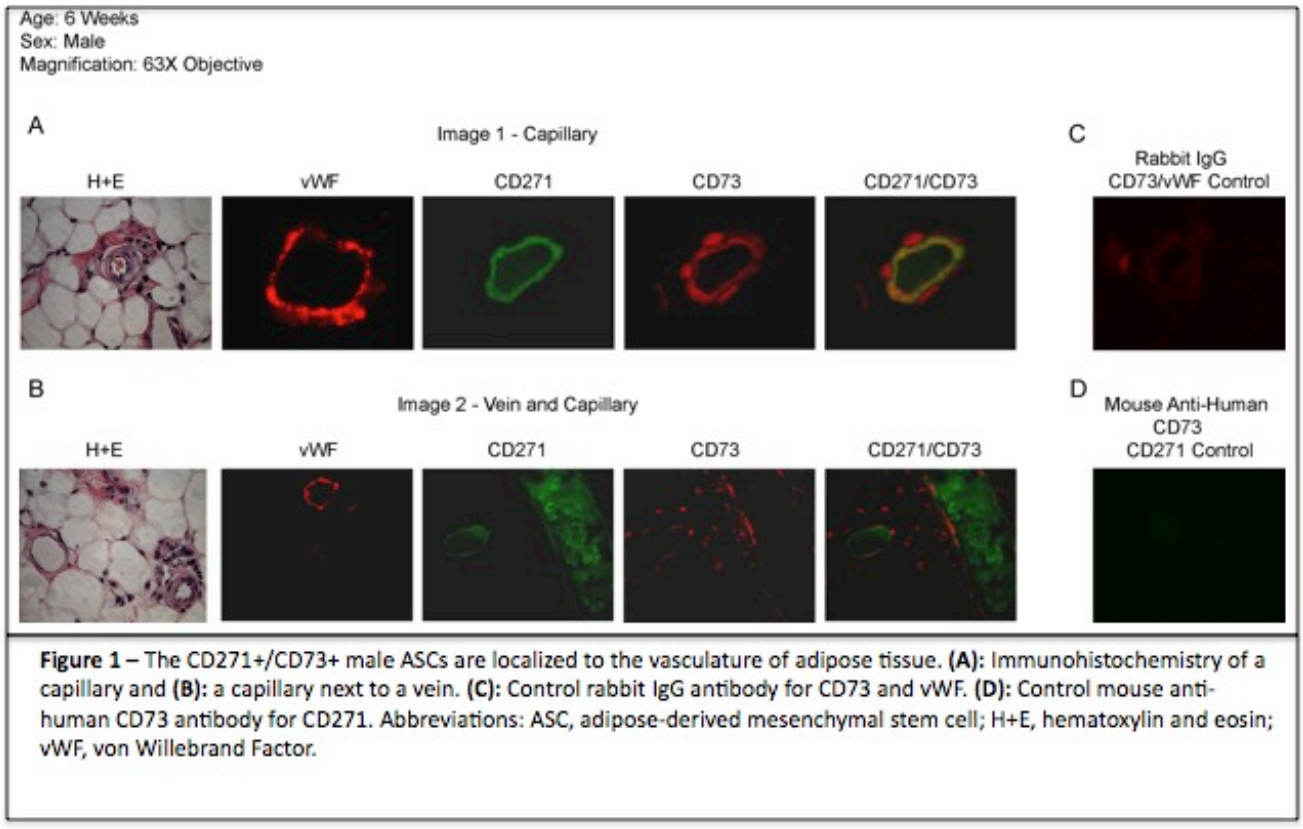


Figure 1 – The CD271+/CD73+ male ASCs are localized to the vasculature of adipose tissue. **(A):** Immunohistochemistry of a capillary and **(B):** a capillary next to a vein. **(C):** Control rabbit IgG antibody for CD73 and vWF. **(D):** Control mouse anti-human CD73 antibody for CD271. Abbreviations: ASC, adipose-derived mesenchymal stem cell; H+E, hematoxylin and eosin; vWF, von Willebrand Factor.

Flow Cytometry

The Number and Percent of ASCs and OPCs is Age Dependent

The flow cytometry data of the primary adipose tissue showed an age related decrease in the percent of ASCs we were able to retrieve from the SVF (Fig. 2A). The percent of ASCs was higher in the 1M and 3M tissue than the 9M tissue. Additionally, we saw a decrease in the number of ASCs we were able to isolate from the SVF per gram of fat (Fig. 2B). The 1M tissue had significantly more ASCs per gram of fat than the 3M tissue. Additionally, there was an age related increase in the percent of OPCs we were able to isolate from the SVF (Fig. 2C, D). There was a significant increase in the 9M tissue over the 1M and 3M tissue. Although the 9M tissue showed an increase in the number of OPCs

isolated from the SVF per gram of fat, this increase was not significant. The reason for this is that although the 9M animals had more overall adipose tissue in the inguinal fat pad, the number of cells isolated from the SVF per gram of fat was less than the 1M and 3M tissue. (Fig 2E). When we looked at the individual ASC markers we noticed that the percent of cells in the SVF expressing CD271 decreased with age (Fig. 2F). Additionally, OCN, a marker for OPCs increased with age although this was not significant between any of the groups (Fig. 2G). Overall, the percent of ASCs in the SVF decrease with age while the percent of OPCs increases with age.

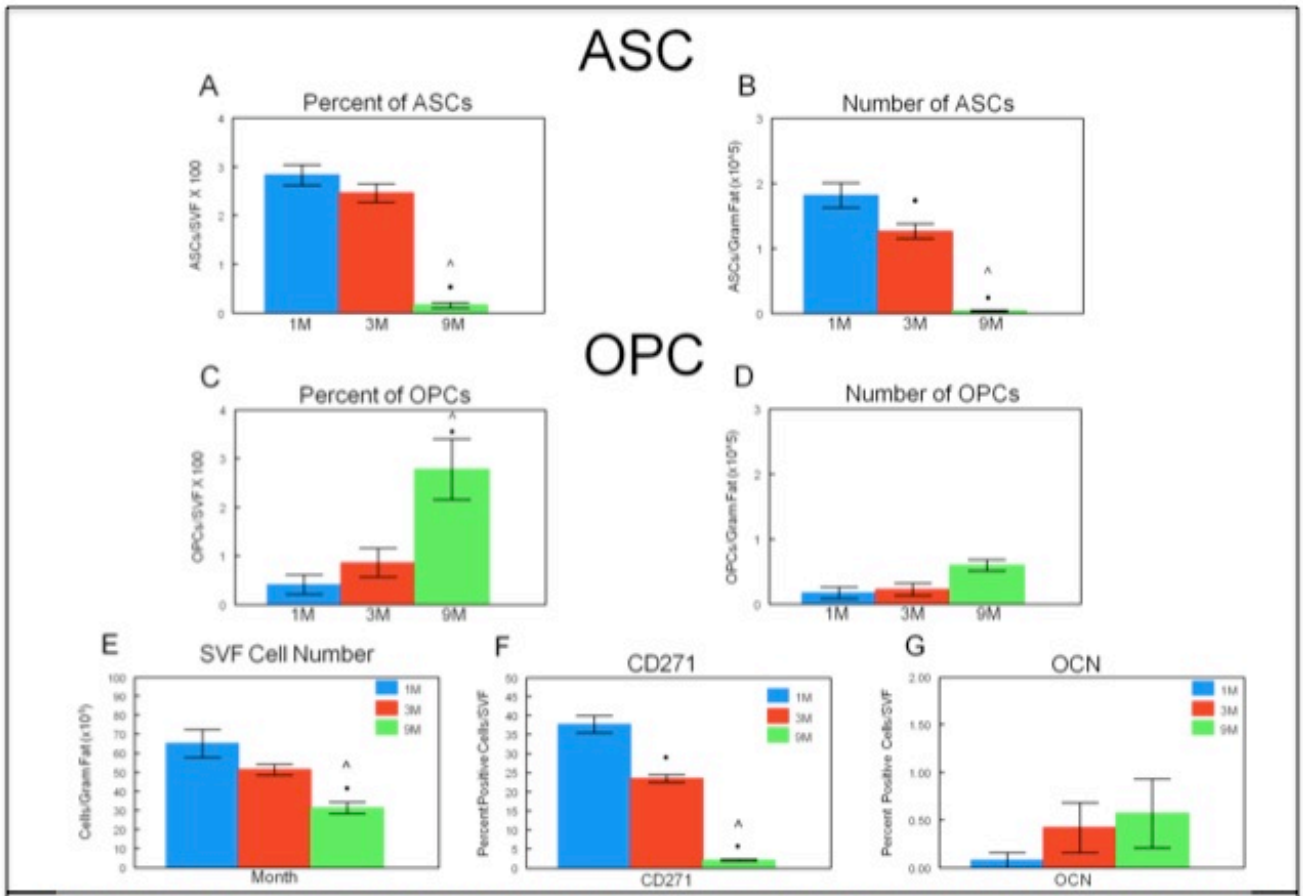


Figure 2 – Age affects the percent and number of ASCs and OPCs in rat adipose tissue. **(A):** Flow cytometry showing the percent of ASCs in adipose SVF by age. **(B):** The number of ASCs per gram of fat. **(C):** Flow cytometry showing the percent of OPCs in adipose SVF by age. **(D):** The number of OPCs per gram of fat. **(E):** The number of cells in the SVF per gram of fat. **(F):** Flow cytometry showing the percent of CD271+ cells in adipose SVF by age. **(G):** Flow cytometry showing the percent of OCN+ cells in adipose SVF by age. All graphs show the mean +/- SEM. •, $p < 0.05$ compared to the 1M cells; ^, $p < 0.05$ compared to the 3M cells. Abbreviations: ASC, adipose-derived mesenchymal stem cell; OPC, osteoprogenitor cell; SVF, stromal vascular fraction

Morphology and Cell Number

After the initial incision, we noticed that the young and old animals had different colored adipose tissue (Fig. 3B). The younger animals (6 weeks) displayed white adipose tissue (WAT) while the older animals had brown adipose tissue (BAT) as shown by the arrows. Additionally, the amount of adipose tissue in the inguinal fat pad increased in the older animals (data not shown). When we plated the cells in culture we noticed a decrease in the cell number for the 1M, 3M, and 9M cells when treated with the osteogenic media at 7 and 14 day (Fig. 3A). Additionally, when the cells were treated with GM, the 9M cells had a lower cell number than the 1M and 3M cells at 7 and 14 days, indicating a decrease in proliferation. When the cells were examined at 14 days, there was a distinct difference in the morphology of the cells grown in GM and OM (Fig. 3C). The cells grown in GM were compact, grew on top of one another, and retained a fibroblast-like morphology consistent with previous stem cell characterizations. The cells grown in OM were larger and more spread out. Additionally, within each media condition there was no noticeable difference in the morphology of the 1M, 3M, and 9M cells. All the cells treated with the GM had the same morphology, and all the cells treated with the OM had the same morphology.

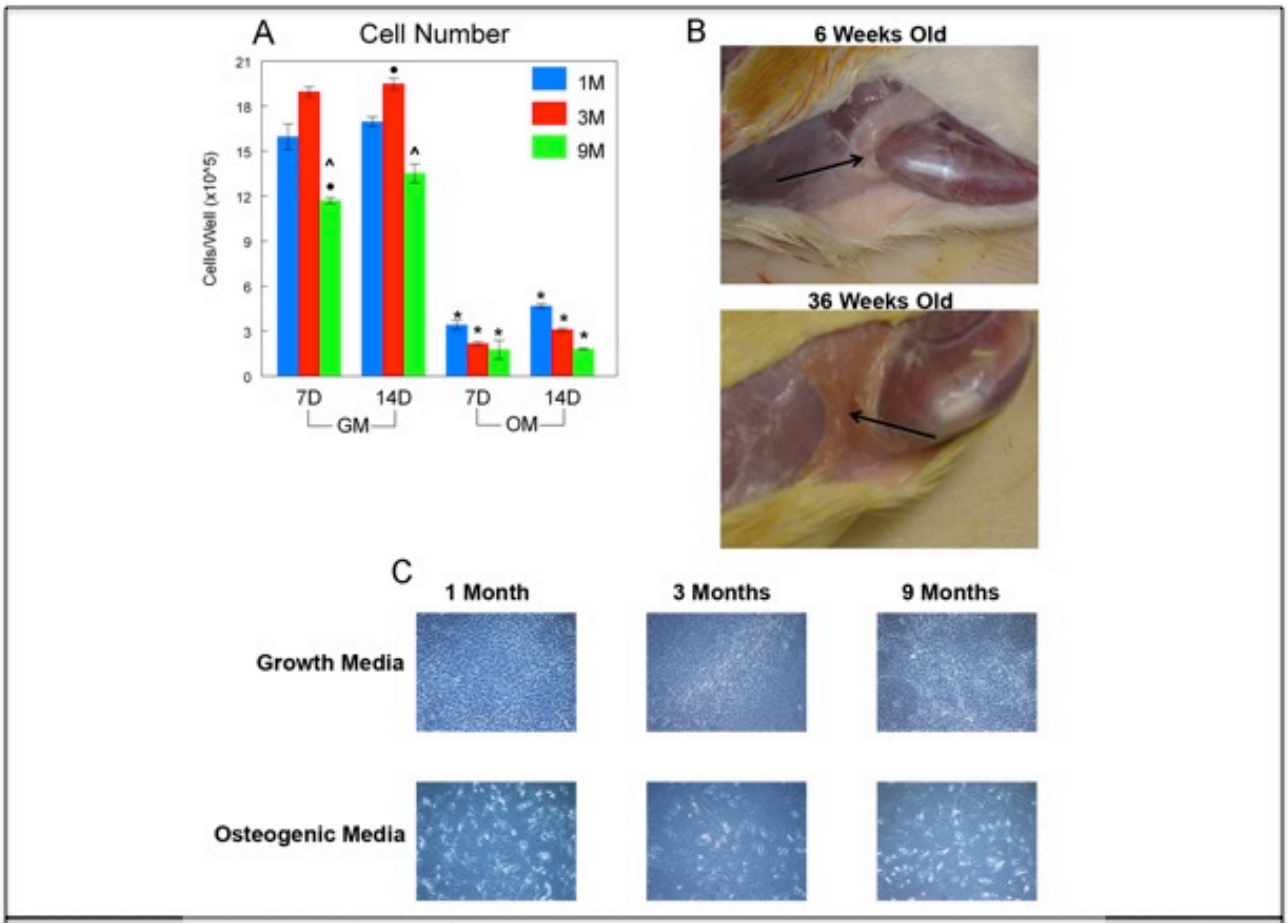


Figure 3 – The ASC tissue morphology and cell number changes with age while the cell morphology does not. **(A):** The cell number of 1M, 3M, and 9M cells treated with GM and OM for 7 and 14 days. **(B):** Digital photographs of the inguinal fat pad from 6-week and 36-week old male Sprague-Dawley rats. The arrows point to the inguinal fat pad. **(C):** Light microscopy images of 1M, 3M, and 9M cells grown on TCPS for 14 days in either GM or OM. Graph A shows the mean +/- SEM. •, $p < 0.05$ compared to the 1M cells; ^, $p < 0.05$ compared to the 3M cells; *, $p < 0.05$ compared to age and time matched cells grown in the GM. Abbreviations: GM, growth media; OM, osteogenic media; TCPS, tissue-cultured polystyrene; M, month; D, day

Biochemical Markers of Osteogenesis

Protein Markers of Osteogenesis are Age Dependent

When the cells were treated with OM for 7 and 14 days there was an increase in the ALPase specific activity for the 1M and 9M cells (Fig. 4A). There was no change in the ALPase specific activity for the 3M cells in either media or time point. There was also an increase in the OCN level for the cells treated with the OM at 7 and 14 days (Fig. 4B). Furthermore, at both 7 and 14 days in the OM

the 3M and 9M cells had higher levels of OCN than the 1M cells. The 1M, 3M, and 9M cells responded to the OM in a similar manner. (Fig. 4C). All the cells produced less OPG in the OM at 7 days. The 1M and 9M cells also produced less OPG in the OM at 14 days, but the level of OPG for the 3M cells did not change significantly at 14 days.

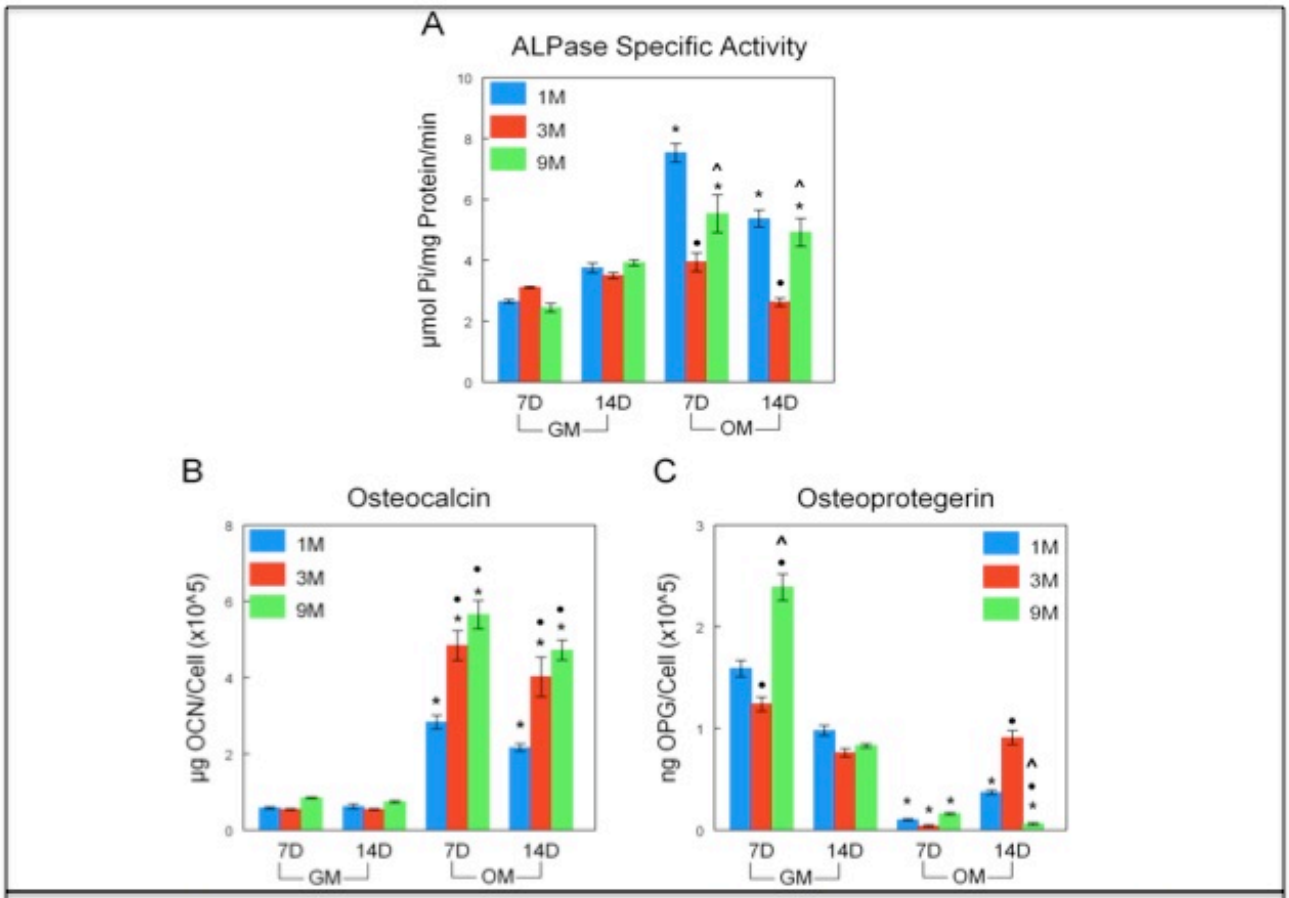


Figure 4 – Biochemical markers of osteogenesis are age dependent. 1M, 3M, and 9M cells were treated with either GM or OM for 7 and 14 days and then measured for **(A)** the ALPase specific activity, **(B)** the level of OCN in the media, and **(C)** the level of OPG in the media. All graphs show the mean +/- SEM. •, $p < 0.05$ compared to the 1M cells; ^, $p < 0.05$ compared to the 3M cells; *, $p < 0.05$ compared to age and time matched cells grown in the GM. Abbreviations: GM, growth media; OM, osteogenic media; ALPase, alkaline phosphatase; OCN, osteocalcin; OPG, osteoprotegerin; M, month; D, day

Real-Time PCR for Markers of Osteogenesis

Genetic Markers of Osteogenesis are Age Dependent

The gene analysis showed that all the cells expressed the early markers of osteogenesis - RUNX2 and Alkaline Phosphatase - as well as the late markers - Bone Sialoprotein and Osteocalcin. There was very little fluctuation in the transcript levels of RUNX2 in both media conditions at 7 and 14 days (Fig. 5A). The ALPase gene expression increased for all the cells at 7 days in the OM, but this was only significant for the 1M cells (Fig. 5B). The 1M cells also increased their ALPase expression over the 3M and 9M cells at 7 days in the OM. Additionally, at 14 days there was a decrease in the expression of ALP for the 1M and 3M cells treated with OM. The gene analysis for OCN showed a slight increase in the expression of OCN when all the cells were treated with OM (Fig. 5C). The expression was significant for the 9M cells at both 7 and 14 days, and this trend is similar to what was seen in the protein expression (Fig. 4B). The cells also showed an increase in the expression of BSP when treated with the OM for 7 and 14 days (Fig. 5D). At 7 days this increase was significant for the 1M and 9M cells, and at 14 days it was significant for the 3M and 9M cells.

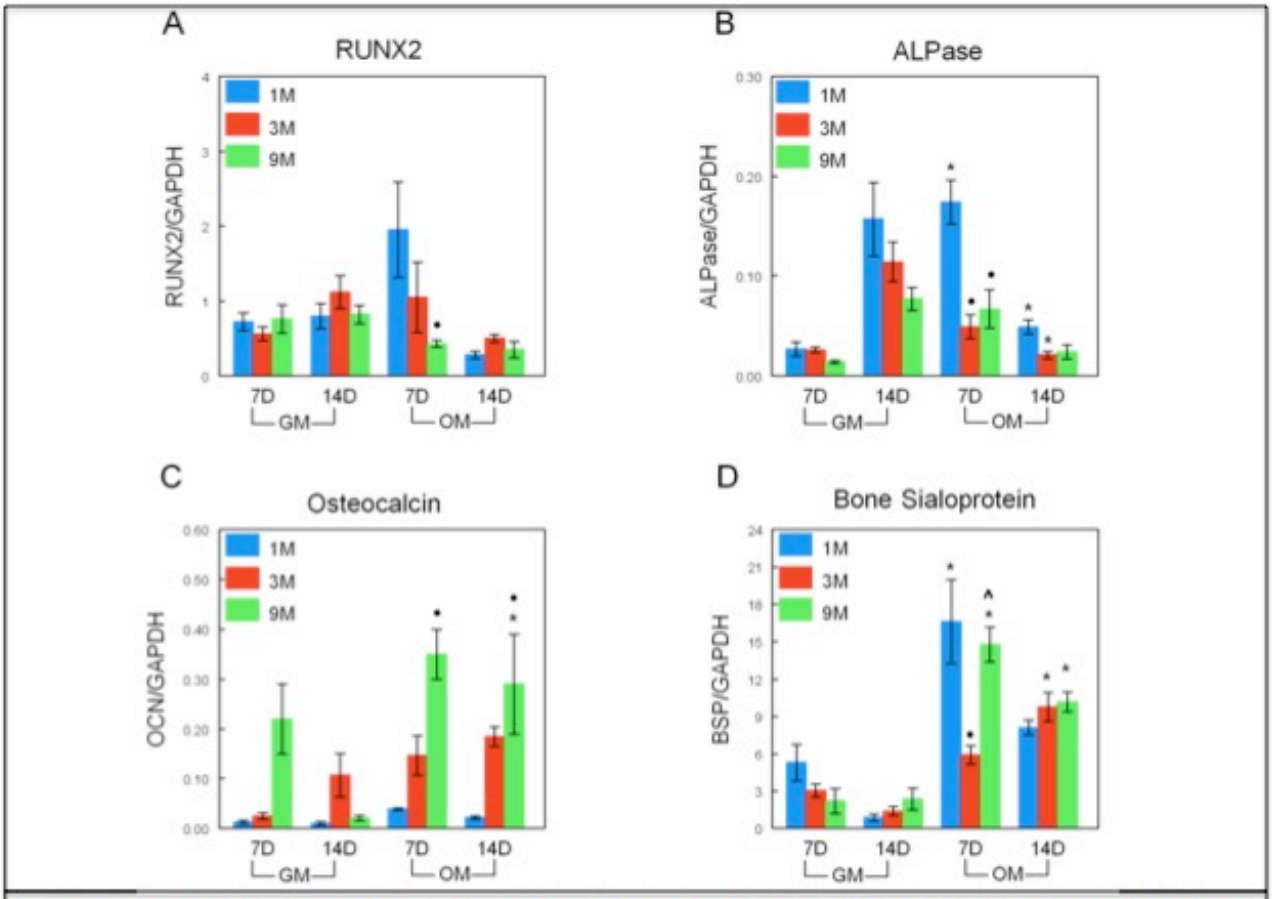


Figure 5 – Real-Time PCR showing that the gene expression of osteogenic markers is age dependent. 1M, 3M, and 9M cells were treated with either GM or OM for 7 and 14 days and then the gene expression was measured for **(A)** RUNX2, **(B)** ALPase, **(C)** Osteocalcin, and **(D)** Bone Sialoprotein. All graphs show the mean +/- SEM. •, $p < 0.05$ compared to the 1M cells; ^, $p < 0.05$ compared to the 3M cells; *, $p < 0.05$ compared to age and time matched cells grown in the GM. Abbreviations: GM, growth media; OM, osteogenic media; RUNX2, runt-related transcription factor 2; ALPase, alkaline phosphatase; M, month; D, day

DISCUSSION

The result of the adipose tissue flow cytometry indicates that the number and percent of ASCs decreases as the animal ages. Although how we measured stem cells is unique, our work is in agreement with other studies. Using a colony forming unit-fibroblast assay, Stolzing et al. found an age related decline in the number of bone marrow stem cells (BMSC). Although BMSCs and ASCs are isolated from different anatomical sources, both of these cell types are mesoderm-derived stem cells. When we broke down the ASC markers we saw an age related decrease in the expression of CD271. This may indicate that CD271 selects for a more homogenous population of ASCs as some recent studies have indicated. Our results indicate that a reduction in the mesenchymal stem cell source may partially explain the decreased ability of our bodies to regenerate damaged tissue as we age. It is important to note that both the number and percent of ASCs decreased with age. This is important because the older animals had more inguinal adipose tissue. However, even if we included the additional adipose tissue it is still likely that the younger animals would have had more total ASCs.

We also investigated how age affects the number of OPCs in adipose tissue. Our flow cytometry showed that the percent of OPCs increases as the animal ages. However, the results were not significant when looking at the number of OPCs because the younger animals had more cells per gram of adipose tissue. It has been shown that as animals age their adipocytes size increases (Holm et al., 1975) and this may contribute to the lowered cells per gram of fat. It may also indicate that in older animals a higher percent of OPCs does not necessarily correlate with more total OPCs. When looking at the individual markers defining OPCs, the percent of E11 did not fluctuate greatly with age, but the percent of OCN did increase with age. These results are in agreement with the flow cytometry results of Eghbali-Fatoureci et al. who showed that the percentage of OCN+ cells in men increases with age. This group was looking at whole blood samples so it is possible that our OCN+ cells were captured in the adipose

tissue vasculature. This, along with recent studies showing that MSCs capable of osteogenesis reside in the perivascular niche, (da Silva Meirelles et al., 2008; Liu et al., 2009) indicates that our OPCs may evolve from cells embedded in the vasculature of adipose tissue. These cells may reside in the perivascular niche until needed and then respond to endocrine stimuli and migrate through the blood to the source of damaged tissue. If this is the case, the increase in OPCs with age may demonstrate an increase in bone turnover, or that ASCs lose their plasticity with age and begin to enter a more differentiated stage.

Our histology of the adipose tissue showed that the CD73 and CD271 positive ASCs are found in the vascular niche of adipose tissue. This is in agreement with other histological studies showing that ASCs are in the vasculature (Yamamoto et al., 2007; Zannettinno et al., 2008). This assessment fits because the vasculature is part of the stromal vasculature fraction that is extracted from the adipose tissue. It also suggests that the stem cells would have direct contact with chemical cues required to activate them for migration and differentiation. It would also imply that the ASCs could be used to repair damage to adipose tissue as well as other mesoderm-derived tissue throughout the body. Whether the ASCs stay within adipose tissue or are in constant fluctuation throughout the body still remains a mystery. Perhaps these mesenchymal stem cells that are isolated from a variety of tissues can move from one tissue source to another when needed. Addressing this question is important because if these stem cells are able to transport from one tissue to another then the adipose tissue itself may vary in the number of ASCs depending on the health of the individual. Finding a way to attract the stem cells to reside in adipose tissue and thus increase their concentration would be an interesting goal if this were the case.

We also discovered that the 9M cells had a lower cell number in culture, and this was significant at 7 and 14 days in the GM. Several other studies have shown that both ASC and BMSC lose their

adherence in culture and proliferate less (Zhu et al., 2009; Stolzing et al., 2008; Baxter et al., 2004). One possibility is that the older stem cells are less responsive to environmental cues with a reduction in cell signaling as Kasper et al. showed in their paper. Additionally, we noticed that the tissue morphology between the younger and older animals was different, with the younger animals displaying WAT and the older animals having BAT. While most noticeable BAT depots in humans disappear in adulthood, BAT remains in the rodent throughout its lifespan (Avram et al., 2007). Unlike WAT, BAT's primary purpose is not to store excess energy, but rather it is used to release chemical energy in food as heat. In older rodents BAT helps to regulate the body temperature, and although BAT may not be seen in adult humans, recent reports suggest that it still exists scattered within WAT. This is important because if rodent BAT has a lower percentage of ASC in the inguinal fat pad, different adipose sites in humans may also fluctuate in their percentage of ASC. If this is the case, it will be important to find the adipose depots in humans that yield the largest percentage of ASCs, especially for the older population.

When the ASCs were treated in culture with OM they increased their ALPase specific activity and OCN secretion. This demonstrates that regardless of age the ASCs have the potential to be pushed towards the osteoblast lineage since ALPase activity is an early marker and OCN a late marker of osteogenesis. These results agree with several papers (Zuh et al., 2009; Shi et al., 2005) showing that young and old ASCs have the same osteogenic potential. Additionally, all three ages showed a significant increase in BSP mRNA in the OM. Since BSP has a high affinity for collagen and has been shown to help during the early hydroxyapatite nucleation (Harris et al., 2000), it can be considered a late marker of osteogenic differentiation. This would imply that the ASCs are not only forming the extracellular matrix, but they are also beginning to mineralize their matrix at 14 days. We did not see an increase in RUNX2 expression when the cells were treated with OM, but this is not surprising. Previous research has shown that the mechanisms pushing a mesenchymal stem cell towards the osteoblast

lineage are tightly regulated, especially RUNX2 expression and protein activity (Bialek et al., 2004). RUNX2 is required to push the cell towards a pre-osteoblast stage but then must be inhibited by Snail1 for the cell to continue towards matrix maturation. Since our cells have already begun to increase their secretion of OCN at 7 days in OM, it is possible that the up regulation of RUNX2 precedes this time point. Our results are similar to the work of de Frutos et al. who showed that osteoblast precursor cells pushed towards osteogenesis with a similar induction media saw the highest RUNX2 expression within 2 days of induction. By day 4 of induction the mRNA expression had already decreased to the baseline level. Future studies should address this property of RUNX2 regulation and collect RNA at an earlier time point. With that said, the cells did show some differences in their response to the GM and OM. The level of OCN secretion was consistently higher in the older cells when treated with OM. This correlates strongly with our flow cytometry results showing that the 9M cells express a higher percentage of OCN. Our real-time PCR results also support the protein studies with the older cells expressing more OCN mRNA in the OM at 7 and 14 days. Additionally, the cells decreased their production of OPG in the OM. Although OPG can be used as a marker for osteogenesis, it has been shown (Kondo et al., 2008) that glucocorticoids like dexamethasone can decrease OPG production by either inhibiting c-Jun phosphorylation or down regulating β-catenin. Even though this was tested in bone-marrow derived osteoblastic/stromal cells, since our cells are a type of MSC and our OM media contains dexamethasone, it is possible that the same mechanisms are in play here.

The biochemical and mRNA assays we performed indicate that the older ASCs retain the same potential for osteogenesis as the younger ASCs. This leads to the question of why the potential to regenerate damage tissue decreases as we age. As shown in figure 2, part of the reason could be a decrease in the number of stem cells as we age. Additionally, extrinsic factors discussed by Conboy et al. could also come into play. In their studies that focus on parabiotic mice, they demonstrate that the

stem cell differentiation potential remains in tact, but the extrinsic cues required to activate the cell and initiate migration are altered as we age. If this is the case, then it might be both a decrease in stem cell number and a change in the systemic environment that contributes to our loss in regenerative capacity as we age.

CHAPTER 3

SEX AFFECTS THE NUMBER OF ASCs IN THE INGUINAL FAT PAD BUT NOT THEIR OSTEOGENIC DIFFERENTIATION POTENTIAL

INTRODUCTION

As was shown in chapter 2, our knowledge of ASCs needs to go beyond the cell and also focus on the individual. We demonstrated that the age of the individual affects both the number of stem cells and also their response in culture. Another factor that must be taken into consideration is the sex of the individual. This is important because men and women distribute adipose tissue differently. Women primarily accumulate adipose in their hips, while men predominantly deposit it in their abdomens. Furthermore, several researchers have demonstrated that ASCs from one adipose source does not necessarily have the same osteogenic potential as ASCs from another source. Taking this into consideration, it would not be surprising to discover that the best site for future male and female ASC harvest does not come from the same anatomical area.

The goal of this study was to examine how sex affects the number of ASCs and their osteogenic potential. Additionally, we harvested ASCs from two different aged male and female rats. Since adipose number and distribution changes with age (Zamboni et al., 1997), our study will allow us to examine any sex-based differences over time. We hope that this and future studies will allow researchers to develop an ideal stem cell harvest protocol not only based on age, but also based on the sex of the patient.

RESULTS

This study showed us that the number and percent of ASCs was not sex dependent at 3M or 9M. The number and percent of OPCs was not sex dependent at 3M, but it was at 9M. At 9M the number of OPCs was greater for the female rats than the male rats. When these cells were plated in culture both the males and females at 3M and 9M expressed markers of osteogenesis when inducted with the OM. This indicates that the stem cells from both sexes have a similar capacity to undergo osteogenesis when treated with the right conditions. We did notice some difference in how the cells responded to their media. Most significantly, the female cells maintained their levels of OPG while the males decreased the secretion of this protein in OM.

Flow Cytometry

The Number and Percent of OPCs, but not ASCs, is Sex Dependent

The flow cytometry of the cells extracted from 3M male and female adipose tissue showed no difference in the percent and number of ASCs (Fig. 6A, B). The flow cytometry of the 9M male and female cells also showed no difference in the percent and number of ASCs. Additionally, the amount of ASCs in the 9M tissue was almost nonexistent, and much lower than the results for the 3M tissue. This result agrees with the flow cytometry in Figure 2. The flow cytometry for OPCs showed no difference in the percent or number when comparing the 3M male and female cells (Fig. 6C, D). However, the number of OPCs in 9M tissue was significantly higher for the cells extracted from the female rats. The percent was also higher in the females although this number was not significant.

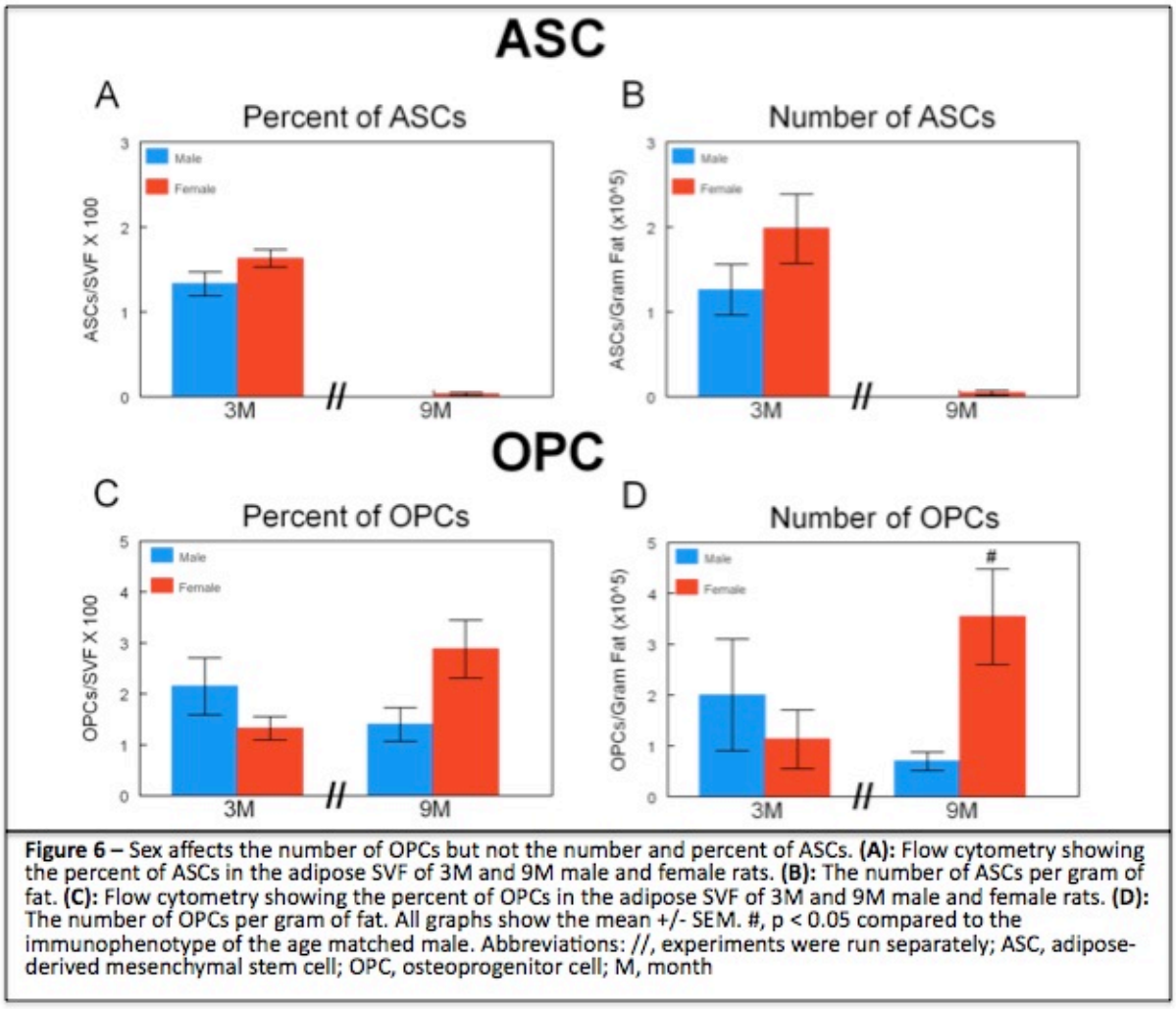
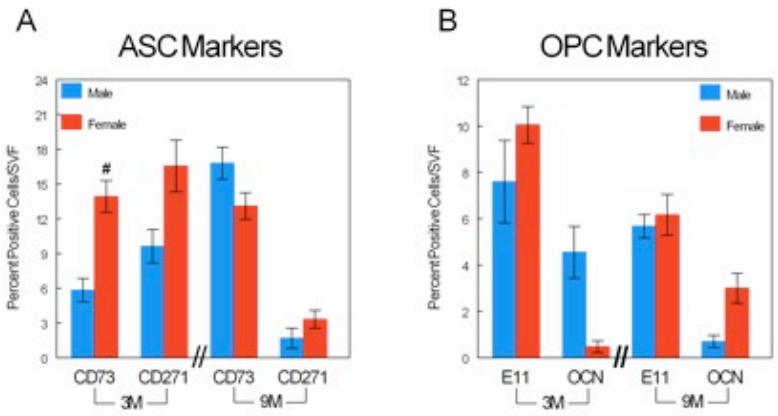


Figure 6 – Sex affects the number of OPCs but not the number and percent of ASCs. **(A):** Flow cytometry showing the percent of ASCs in the adipose SVF of 3M and 9M male and female rats. **(B):** The number of ASCs per gram of fat. **(C):** Flow cytometry showing the percent of OPCs in the adipose SVF of 3M and 9M male and female rats. **(D):** The number of OPCs per gram of fat. All graphs show the mean +/- SEM. #, $p < 0.05$ compared to the immunophenotype of the age matched male. Abbreviations: //, experiments were run separately; ASC, adipose-derived mesenchymal stem cell; OPC, osteoprogenitor cell; M, month

To elucidate the reasons for any age or sex related difference in ASC or OPC percent, we looked at each cell population by their individual markers. When we examined the 3M ASC population we saw that a higher percent of cells were CD271 positive than CD73 positive (Fig. 7A). Additionally, although the cells from female tissue exhibited a higher percent of CD73 and CD271, there was no difference in the percent of ASCs between the two sexes at 3 months (Fig. 6A). When we looked at the 9M ASC population we saw that a higher percent of cells were

CD73 positive than CD271 positive, the opposite of the 3M results (Fig. 7A). The decrease in CD271 could be responsible for the lower ASC percent in the 9M cells.

We also looked at the 3M and 9M OPC population by OCN and E11 alone. Although the percent of E11 did not fluctuate much by age or sex, the percent of OCN did change (Fig. 7B). In the 3M cells the percent of OCN was higher in the male cells, whereas in the 9M cells the percent of OCN was higher in the female cells. This could have caused the significant increase in the number of OPCs in the 9M female cells over the male cells (Fig. 6D).



C

Immunophenotype (%) of Male and Female Cells in Culture

			7 Days				14 Days			
			CD73	CD271	E11	OCN	CD73	CD271	E11	OCN
3M Cells	GM	Male	92.9	0.5	0	0	61	8.7	1	0.7
		Female	91	0.9	0	0	60.1	10.3	0.2	0.2
	OM	Male	25.8	14.8	0.1	0	4.7	4.2	2	0.1
		Female	22.7	8.1	0	0	2.2	1.9	2.6	0
9M Cells	GM	Male	45	6	1.3	0.2	19.5	9.1	1.2	0.6
		Female	40.3	3.4	1.3	0.1	18.2	6.3	1	0.2
	OM	Male	2.2	3.6	1.8	0.3	4.9	2.8	5.5	4.7
		Female	0.2	2.4	3.9	0	3.3	0.9	3.3	1.9

Figure 7 – The immunophenotype of SVF derived cells in primary tissue and in culture. Flow cytometry showing the expression of **(A)** CD73 and CD271 as well as **(B)** E11 and OCN in 3M and 9M male and female cells extracted from the SVF of adipose tissue. **(C)** 3M and 9M male and female ASCs were treated with either GM or OM for 7 and 14 days and then flow cytometry was used to assess the percent of cells expressing CD73, CD271, E11, and OCN. Graphs A and B show the mean +/- SEM. #, $p < 0.05$ compared to the surface marker expression of the age matched male. Abbreviation: ASC, adipose-derived mesenchymal stem cell; OPC, osteoprogenitor cell; SVF, stromal vascular fraction; GM, growth media; OM, osteogenic media; OCN, osteocalcin; M, month; D, day

Biochemical Markers of Osteogenesis

Cell Number and Protein Markers of Osteogenesis are Sex Dependent

The male and female cells responded to the conditioned media in a similar fashion. In the GM they had a higher cell number than when treated with the OM (Fig. 8A, B). However, we did observe some differences in how the male and female cells responded to their media. The male cells had a higher cell number in the GM than the female cells. This was significant for the 3M cells but not for the 9M cells. When the cells were treated with OM, the female cells appeared to have a higher cell number for both ages and time points, but this difference was not significant.

When the male and female cells were treated with the OM there was an increase in the ALPase specific activity (Fig. 8C, D). In the 3M cells this increase was only observed at 7 days for the female cells, whereas in the 9M cells the increase was seen in both sexes at both time points. Additionally, the 3M female cells displayed a significantly higher level of ALPase specific activity over the males when treated with the OM for 7 days. When looking at the OCN levels, the male and female cells responded in a similar fashion and secreted more OCN when treated with the OM at 7 and 14 days (Fig. 8 E, F). This trend was seen for both the 3M and 9M cells. Finally, the male and female cells responded differently to the media in their secretion of OPG (Fig. 8G, H). The male cells decreased their OPG secretion when treated with the OM for 7 days. Only the 3M female cells decreased their OPG secretion when treated with the OM for 7 days. The 9M female cells did not show a significant change in the OPG levels when treated with either media. Furthermore, female cells secreted more OPG than the male cells. The 3M female cells produced significantly higher levels of OPG when treated for 7 and 14 days in both medias. The 9M female cells produced more OPG when treated with the OM for 7 and 14 days.

Therefore, the female cells generally had a higher level of OPG when treated with both the GM and OM.

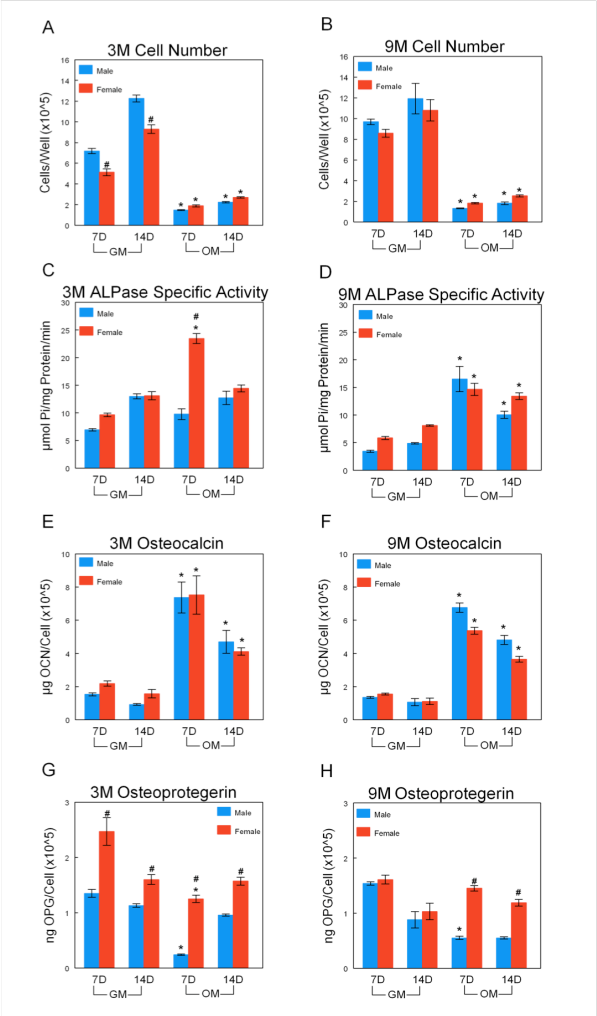


Figure 8 – Cell number and biochemical markers of osteogenesis are sex dependent. Male and female cells extracted from the SVF of the rat inguinal fat pad were treated with either GM or OM for 7 and 14 days and then measured for (A,B) 3M and 9M cell number (C,D) 3M and 9M ALPase specific activity (E,F) 3M and 9M osteocalcin level, and (G,H) 3M and 9M osteoprotegerin level. All graphs show the mean +/- SEM. *, $p < 0.05$ compared to the sex and time matched cells grown in the GM; #, $p < 0.05$ compared to the time and media matched male. Abbreviations: SVF, stromal vascular fraction; ALPase, alkaline phosphatase; GM, growth media; OM, osteogenic media; M, month; D, day

Real-Time PCR Markers of Osteogenesis

Genetic Markers of Osteogenesis are Sex Dependent

Similar to the results in Fig. 5, we saw very little fluctuation in the RUNX2 expression for the 3M male and female cells (Fig. 9A). Both sexes showed an increase in ALPase expression when treated with the osteogenic media (Fig. 9B). At 14 days in the OM the male cells showed an increase while the females showed a significant increase. At this time point the expression of ALPase was significantly higher for the females than the males. This agreed with our protein studies from Fig. 8. For the OCN expression there was not a significant fluctuation at any time point (Fig. 9C). However, at 14 days in the OM there appeared to be an increase over the GM for both the males and females. Finally, there was a significant increase in the BSP expression for the 3M male and females when treated with OM for 14 days (Fig. 9D). Additionally, at this time point the female cells express more BSP than the male cells.

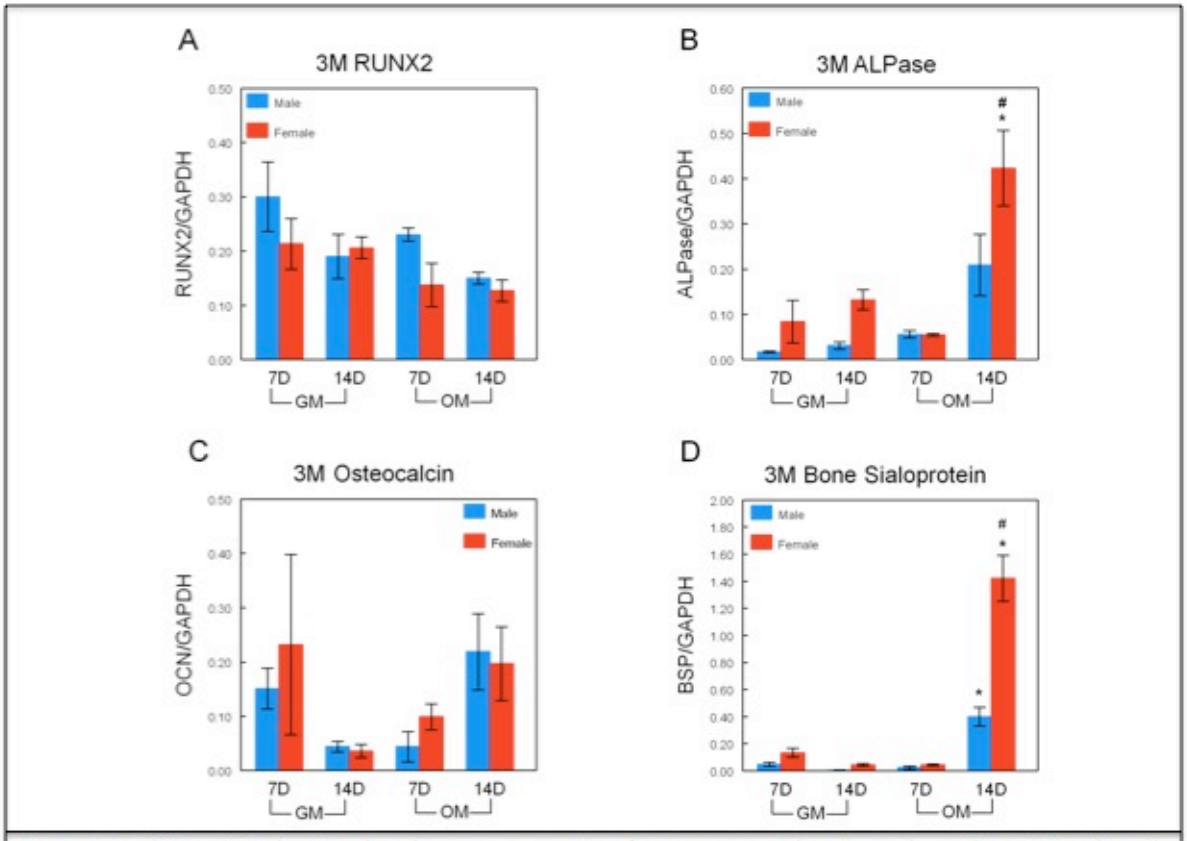


Figure 9 - Real-Time PCR showing that the gene expression of osteogenic markers is sex dependent. 3M male and female cells were treated with either GM or OM for 7 and 14 days and then the gene expression was measured for **(A)** RUNX2, **(B)** ALPase, **(C)** Osteocalcin, and **(D)** Bone Sialoprotein. All graphs show the mean +/- SEM. *, $p < 0.05$ compared to sex and time matched cells grown in the GM; #, $p < 0.05$ compared to the time and media matched male. Abbreviations: GM, growth media; OM, osteogenic media; RUNX2, runt-related transcription factor 2; ALPase, alkaline phosphatase; M, month; D, day

DISCUSSION

The second aim focused on the role sex plays in ASC number and osteogenic differentiation potential. We chose to focus our study on 3M and 9M male and female rats. We chose these ages because at 3M the rats are just starting their sexual maturity, and at 9M they have stopped their skeletal development. Due to these circumstances, we believe that the adipose microenvironment and hormonal balance would be most different at these ages. We saw from our flow cytometry that the number and percent of ASCs in the inguinal fat pad is the same for

3M and 9M male and female rats. We also noticed that the percent and number of ASCs for the 9M rats was almost nonexistent, and this further validates our finding in the first study. When we broke the phenotypes into their individual markers, we saw a large reduction in the percentage of CD271 positive cells in the 9M rats. Recent reports have provided evidence that CD271 is a specific marker of ASCs. Bone marrow and adipose tissue isolates selected for CD271 yielded a subset of cells with a higher proliferative and osteogenic differentiation capacity than the CD271 negative population (Yamamoto et al., 2007; Quirici et al., 2009). This would indicate that CD271 selects for a more homogenous ASC population, and that the reduction of CD271 in our 9M cells indicates a lower percentage of ASCs in the rat inguinal adipose tissue. When we looked at the number and percent of OPCs we saw no differences at 3M, but at 9M the number of OPCs is significantly higher in the female rats. Looking at figure 7 we can see that the percent of E11 is the same at 9M, however, the females express more OCN. Several studies have indicated that OCN levels are inversely related to bone mineral density (BMD). Also, it is known that OCN increases in women with age, and that older women have an increase in bone turnover and a lower BMD (Lumachi et al., 2009). Additionally, the 9M females had a higher cell yield per gram of adipose tissue (results not shown). The combination of these two can explain the increase in OPCs in the 9M females over the males.

When the cells were plated in culture we saw that both the 3M and 9M male and female rats had a similar tendency to express markers of osteogenesis when given the OM. Both sexes increased their cell number in the GM over the OM, and this occurred at 3M and 9M. When looking at the ALPase activity and the OCN secretion, both sexes increased these osteoblast markers in the OM. For the ALPase activity, the 3M female rats had significantly higher numbers at 7 days in the OM, but this increase was not seen at 9M. For the OCN secretion, both

sexes and ages showed significantly higher numbers in the OM at 7 and 14 days, but there was no difference between the sexes. These results for the female ASCs agree with the work of Zuh *et al*. showing that female human ASCs retain their osteogenic potential with age. Although the 3M and 9M female cells were not run together, both cells responded to the OM and increased their ALPase activity and OCN secretion. The real-time PCR further supports the protein studies by showing an increase in ALPase, OCN, and BSP mRNA at 14 days when the 3M male and female cells were treated with OM, although the results are not significant for OCN. Our results for the sex study tend to disagree with the results of Aksu *et al.* showing that adipose tissue from males has a higher osteogenic potential than females. Our two studies have several differences though, most importantly is their use of abdominal subcutaneous tissue from humans, while we used tissue from the inguinal fat pad of rats. Their results indicate that male adipose tissue has a higher osteogenic potential and that adipose from the superficial layer has a higher differentiation potential than adipose from the deep layer. They speculated that this might be due to the increased vasculature of the superficial adipose tissue, and this would be in agreement with the results of Peptan *et al.* Although it is hard to compare our two studies, it is important to remember that men and women distribute adipose tissue to different regions. Therefore, one source of tissue might have a higher osteogenic potential in men, while another source would have a higher osteogenic potential in women. It will be important to study the ASC potential from different anatomical sources and sexes since any differences will help optimize future ASC therapies. Finally, we saw a large difference in the secretion of OPG between the sexes in both the 3M and 9M cells. While the male ASCs decreased their production of OPG in the OM, the female cells were able to retain their levels in both medias. It has been demonstrated that estrogen can up regulate the production of OPG, an inhibitor of osteoclastogenesis (Szulc et al.,

2008). Additionally, studies have shown that rat and human female adipose tissue, including cells in the stromal vascular fraction, express the estrogen receptor (ER) (Mizutani et al., 1994). We also known that the phenol red used in our media is able to mimic the effects of estrogen (Bindal et al., 1988). Given this information, we believe that the media may have played a role in stimulating the ERs of female ASCs to maintain their production of OPG in the OM. This is important because OPG plays a key role in preventing osteoclast maturation by binding the RANKL, and so ASCs expressing this protein might be able to better promote bone formation by inhibiting bone loss *in vivo.*

Our study is important for clinicians using ASCs because we demonstrate that the osteogenic potential of male and female ASCs is similar, although differences do exist to how they respond in culture. With that said, we realize that there are limitations to this work. One of the major obstacles is the source of adipose tissue, since all the adipose tissue was taken from the same site, the inguinal fat pad. Future studies that address sex-based ASC differences should compare these stem cells from different anatomical sources in men and women. This will allow researchers to examine the possibility that the ideal source of ASCs from one sex is not the same as in the other sex. It will also ensure that the optimal isolation procedures are in place so that we can yield the maximum amount of ASCs with the best osteogenic potential from each patient.

CONCLUSION AND CLINICAL IMPLICATIONS

This work helps shed light on the ASC location, as well as the number and osteogenic differentiation potential in regards to age and sex. In aim 1 we discovered that the cells positive for CD73 and CD271 were found in and around the vasculature. Most likely these cells reside in the vasculature niche of adipose tissue until a chemical signal triggers them for migration. The

vasculature would afford them the easiest opportunity to migrate to a site within adipose tissue and around the body. Perhaps these ASCs can both originate from other sources like the bone marrow, and also contribute to the regeneration of mesoderm derived organs around the body.

We also discovered that the number of ASCs decreases with increasing age. This could help explain why our ability to regenerate tissue decreases as we age. As we age our partially exhausted source of stem cells impairs our ability to fix damaged tissue in a timely and efficient manner. Clinicians may need to isolate more adipose tissue from older patients, or expand their number in culture, to yield the appropriate number of stem cells. Additionally, in aim 1.3 we discovered that once placed in culture and feed with an osteogenic media, age did not affect the potential of the ASCs to differentiate down the osteogenic lineage. This would imply that the intrinsic ability of the stem cell is maintained with age and indicate that extrinsic factors could play a role in stem cell potential with age. As I pointed out earlier, several authors have shown that extrinsic chemical cues needed to trigger the stem cells for migration and differentiation are either inhibited or not present with increasing age. It could be both a decrease in stem cell number as well as a reduction in the extrinsic factors needed for stem cell migration that cause the decrease in tissue regeneration with age.

In aim 2 we investigated how sex affects the ASC number and osteogenic potential. We saw that male and female ASCs at 3 and 9 months have a similar number of stem cells in the adipose tissue. Additionally, when grown in culture the males and females maintain a similar osteogenic differentiation potential. The main difference was that the 3M and 9M female ASCs had higher levels of osteoprotegerin in both the GM and OM. This may indicate that the female ASCs could help prevent bone loss *in vivo* by their increased production of OPG. Future studies should address this phenomenon by studying the difference in the number of steroid receptors

between male and female ASCs. Perhaps the female ASCs have a higher number of estrogen receptors which may have helped them maintain their levels of OPG in the presence of dexamethasone, a known inhibitor of OPG. Furthermore, because male and female humans have a different distribution pattern of adipose tissue, it will be important to see how location affects ASC number. Previous studies indicate that location does affect the osteogenic potential of the ASCs, and so the optimal site for ASC retrieval between men and women may be different.

This work provides clinicians with additional insight into the properties of ASCs. It is important because these stem cells will be targeted to people of different ages, sexes, and medical conditions. Understanding ASC number and potential in regard to these factors will help clinicians optimize their patient's ASC therapy.

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